The Effect of Transglutaminase Crosslinking on the Rheological Characteristics of Heated Peanut Flour Dispersions

G. GHARST, D.A. CLARE, J.P. DAVIS, AND T.H. SANDERS

ABSTRACT: Peanut flour (PF) is a high-protein ingredient prepared after the partial extraction of oil from roasted peanut seed. Microbial transglutaminase (TGase) catalyzes protein crosslinking via acyl-transfer reactions, resulting in the modification of functional properties such as viscosity, gelation, solubility, and water holding capacity. This work was conducted to observe changes in rheological properties of PF dispersions in the presence and the absence of TGase and amidated pectin (AP). Dispersions were characterized across a range of conditions, including controlled heating and cooling rates under both large- and small-strain deformations. Gelation occurred at temperatures above 78 °C using PF dispersions treated with TGase compared to untreated dispersions devoid of the enzyme (about 68 °C). The addition of AP (0.5%) resulted in a general increase in viscoelasticity for all dispersions. AP addition also minimized the shift in gel point temperature caused by TGase polymerization reactions. High-molecular-weight polymers were formed in TGase-treated PF dispersions in both the presence and the absence of AP; however, polymer formation was more rapid in PF dispersions without AP. Ortho-phthaldialdehyde assays indicated about 40% protein coupling in PF dispersions treated with TGase compared to about 20% in those containing both AP and TGase. Collectively, these data suggest potential applications of TGase-treated PF dispersions, both in the presence and the absence of AP, for use in peanut-base food products, including protein bars, shakes, and value-added baked goods.

Keywords: amidated pectin, peanut flour, peanut proteins, rheology, transglutaminase

Introduction

Peanuts (*Arachis hypogaea* L.) are a staple food commodity around the world. In the last few decades, peanut flour (PF) has increasingly been used as a food ingredient. PF is a dry powder manufactured after the partial extraction of peanut oil from roasted peanut seed. PF is typically composed of approximately 40% to 50% protein, 10% to 30% fat, and 20% to 30% carbohydrate, some of that being fiber (Suknark and others 1997), compared to roasted peanuts, which comprise approximately 25% to 30% protein, 50% fat, and 20% carbohydrate/fiber (Hoffpauir 1953; Basha and others 1976).

Heat-induced rheological modifications of protein-based formulations are important to the texture and stability of numerous food products, and previous research has established that protein solutions prepared with peanut protein isolate will form gels under the appropriate conditions. Arachin, the primary protein fraction of peanut seed, forms a heat-reversible gel below pH 3.8 and at concentrations above 7.5% w/v (Kumar and others 1980). Kella and Poola (1985) reported that arachin (15% w/v) forms a gel when heated at 90 °C for 15 min and then cooled to 5 °C for 24 h.

Recently, rheological analyses were conducted for a range of aqueous dispersions prepared from commercially available PFs (Davis and others 2007). Flours varied according to the degree of roast, either light or dark, and by the residual fat content present in the flours, either 12% or 28%. Small- and large-strain rheological measurements were used to characterize aqueous flour dispersions at pH 8.0. Low-fat flours were more viscous than high-fat flours on an equal weight basis. Gelation was also observed in these dispersions as determined by small-strain oscillatory analyses upon heating at 1 °C/min. The 28% fat and/or dark-roast flours formed gels less effectively than the 12% fat and/or light-roast flours, with residual fat content most affecting rheological properties.

Microbial transglutaminase (TGase) catalyzes protein crosslinking through acyl-transfer reactions between glutaminyl residues (donor) and primary amines (acceptor) (Folk and Chung 1985). TGase is used in the food industry to modify a variety of protein-related functional properties, including stability to phase separation, the formation of heat- and water-resistant films, water holding capacity (WHC), protein solubility, and rheological properties such as gelation and viscosity characteristics (Zhu and others 1995; Truong and others 2004). Common substrates for TGase include whey, casein, and soy protein (Schorsch and others 2000; Truong and others 2004; Tang and others 2006). Recently, Clare and others (2007) reported that PF proteins, present in 12% light-roast PF dispersions, were polymerized by microbial TGase as determined by qualitative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitative ortho-phthaldialdehye (OPA assay) analyses. These studies also established that the viscosity of the PF dispersions was lowered after treatment with the enzyme.

Polysaccharides and proteins are commonly used simultaneously in manufactured foods. Both classes of ingredients clearly affect product functionality; however, due to the complexity and the variety of proteins and polysaccharides used in foods, no hard

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and fast rules are established for product functionality containing mixtures of proteins and polysaccharides (Dickinson 2006). Amidated pectin (AP) is a low-methoxy pectin obtained when ammonia is used in the alkaline deesterification process of the pectin molecules. This deesterification results in the replacement of some of the neutral methoxy groups with more polar amide groups. AP is used in the food industry to form gels, increase product viscosity, and perform as a stabilizer (Barrera and others 2002).

Previous data suggested that low-methoxy pectin may form ionic conjugates with specific amino acid groups and/or peptides derived from peanut proteins (Mouecoucou and others 2004). In this event, protein–polysaccharide interactions likely result from electrostatic attractions between the negatively charged polysaccharides and positively charged areas of the protein (Imeson and others 1977; Xia and Dubin 1994). However, to date, the effects of AP on these protein systems have not been investigated. Accordingly, the objective of this research was to evaluate the effect of exogenous TGase on the rheological characteristics of PF dispersions prepared in the presence and the absence of AP.

Materials and Methods

Materials

Light-roast PF with 12% fat was provided by Golden Peanut Company (Alpharetta, Ga., U.S.A.). AP, GENU® Type LM-20AS, was contributed by CP Kelco (Wilmington, Del., U.S.A.). Herein, the AP used in this study had a degree of amidation of 19% and a degree of esterification of 30%. Purified microbial TGase was donated by Ajinomoto Food Ingredients LLC (Japan) and stored at −20 °C prior to use. All other regent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Methods

Hydration of peanut flours and amidated pectin. PF (20% w/w total solids) was dispersed in deionized water with vigorous stirring using a magnetic stir plate. AP was added at a final concentration of 0, 0.5, or 1.0% and the pH of the PF–AP dispersion was adjusted initially to 8.0 with 2 N NaOH, then stirred for 1 h and readjusted to pH 8.0 as necessary.

Transglutaminase activity. TGase was supplied as a powder mixed with maltodextrin. The enzyme was solubilized in 1.0 M Tris-acetate buffer, pH 6.4, and assayed according to the method of Folk and Chung (1985) with minor modification by Truong and others (2004). The purity of TGase was greater than 90% based on SDS-PAGE protein banding patterns (Wilcox and Swaisgood 2002). N-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine were used to measure enzymatic activity at 37 °C. Reagent concentrations in this assay mixture included: 100 mM hydroxylamine, 15 mM CBZ-glutaminyl-glycine, 5 mM calcium chloride, and 10 mM dithiothreitol in 200 mM Tris acetate buffer, pH 6.0. Under these experimental conditions, 1 unit of enzyme activity was defined as an absorbance change of 0.29/min at 525 nm at pH 6.0, forming 1 μmol of hydroxamate per minute. The specific activity of TGase was determined to be 600 units per gram of powder.

Enzymatic polymerization of peanut flour dispersions. TGase was added to PF dispersions (20% w/w, pH 8.0) at a ratio of about 5 units/g PF while AP was initially added at 1.0%. The dispersions were slowly mixed in an incubator at 37 °C and the soluble supernatants were removed after centrifugation at 13600 × *g*. These fractions were evaluated for the degree of crosslinking by means of OPA measurements. Changes in the protein banding pattern after TGase polymerization were accomplished by SDS-PAGE. Aliquots were removed at timed intervals for both types of analyses (0, 3, 6, and 24 h). Control dispersions, without TGase and AP, were evaluated as well.

Rheological analysis. PF dispersions ± AP (0.5% w/w for rheological testing) and ±TGase were incubated at 37 °C while shaking. Control dispersions (minus TGase) were also incubated at 37 °C for equivalent time periods and rheological measurements were initiated immediately after TGase treatment. All rheological measurements were conducted using a stress-controlled rheometer (StressTech; Rheologica Instruments AB, Lund, Sweden), equipped with a CC25 serrated cub and bob geometry with a gap width of 1 mm. Dispersions were added to the cup, such that they covered the bob, and a layer of mineral oil was added over the top of the dispersion to minimize vapor loss. The apparent viscosity of PF dispersions was determined while heating the samples from 40 °C to approximately 90 °C at a rate of 1 °C/min with a rotational shear rate of 50 per second. Small-strain, constant oscillatory testing was performed at a stress level of 1.5 Pa and a frequency of 0.1 Hz during heating of the samples as described. The stress, 1.5 Pa, was identified to be within the linear viscoelastic region (LVER) based on stress sweeps at 10 Hz for all samples at 40 °C. Frequency sweeps from 0.1 to 10 Hz were also applied to samples held at 90 °C. Rheological tests were conducted in duplicate.

Protein concentration measurements. PF (20% solids w/w) ± AP (1.0 % w/w) were prepared in deionized water and incubated at 60 °C for 3 h, then stored overnight at 4 °C. Dispersions were then reheated to 37 °C, and in identified samples TGase was added. All samples were centrifuged at 13600 × *g* for 30 min at room temperature prior to measuring the protein concentrations, according to bicinchoninic acid (BCA) methodologies developed by Pierce Inc. (Rockford, Ill., U.S.A.). All samples were measured in duplicate.

Ortho-phthaldialdehye analysis. All samples were diluted with deionized water and microcentrifuged at 13600 × *g* for 5 min. Twenty microliters of the resultant supernatants were collected, added to 2.0 mL of the OPA reagent, and incubated for 2 min at 25 °C. Absorbance measurements at A_{340} nm were then measured using a Gilford 2600 spectrophotometer (Oberlin, Ohio, U.S.A.). All readings fell within an absorbance range of 0.1 to 1.0 as described by Church and others (1983). Samples were analyzed in triplicate.

SDS-PAGE electrophoresis. PF supernatants were assayed for protein content to ensure that equivalent amounts were loaded into each gel lane. Samples were mixed with 0.9 M Tris sample buffer containing 8% SDS and 5.0% β-mercaptoethanol and heated at 100 °C for 10 min prior to loading onto 10% to 20% Tris-Tricine gradient polyacrylamide gels. After electrophoresis was completed, the samples were stained to visualize the protein banding pattern using a colloidal Coomassie Blue staining reagent (Invitrogen Inc., Carlsbad, Calif., U.S.A.).

Water holding capacity. The WHC of PF dispersions, ±TGase ± AP (0.5% w/w), was determined according to the centrifugation method of Fleming and others (1974) with minor modification by Resch and others (2005). Control- and enzyme-treated dispersions were incubated for 6 h and 24 h at 37 °C, then centrifuged at 635 × *g* per 15 min. Supernatants were carefully removed and the water held per gram of solid was determined by calculated weight differences. Test samples were analyzed in triplicate.

Statistical analysis. Statistical analyses of WHC were analyzed by analysis of variance using SAS statistical software (version 8.0; SAS Institute, Cary, N.C., U.S.A.).

Results and Discussion

Good solubility is critical for proteins in most functional applications, and it was previously observed that protein solubility in aqueous PF dispersions was generally increased upon heating

to 60 °C for about 3 h (Clare and others 2007). However, notable precipitation was observed in dispersions treated with TGase after 24 h, suggesting decreased solubility of extensively crosslinked protein materials (Clare and others 2007). For the current work, the addition of 1% AP into TGase-treated PF dispersions lowered the amount of precipitation seen after 24 h (Figure 1). Perhaps, this observation may result from electrostatic interactions that occur between the PF protein content and the soluble carbohydrate (Mouecoucou and other 2004). Moreover, the degree of catalysis may be lower in the PF–AP dispersion due to increased viscosity of the PF substrate dispersion itself (Larsen and others 1994).

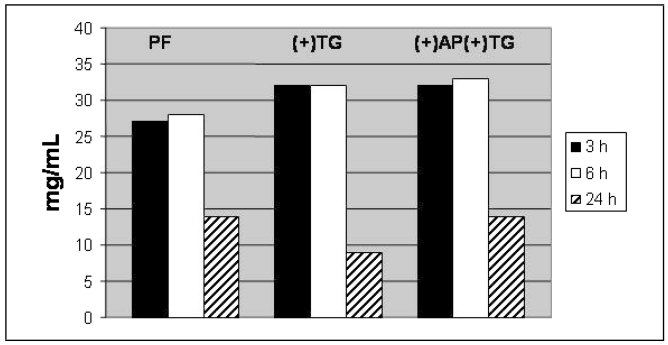

Figure 1—Protein concentration measurements using 20% PF dispersions (w/w) ±1.0% AP (w/w), initially prepared at 60 °C/3 h and stored overnight at 4 °C. Dispersions were reincubated at 37 °C for 3, 6, and 24 h in the presence and absence of TGase, pH 8.0. Reported values are means of duplicate measurements. Bovine serum albumin (2.0 mg/mL) was used as the standard.

SDS-PAGE data confirmed the formation of high-molecular-weight (HMW) protein polymers using PF dispersions that were treated with TGase for 3, 6, and 24 h at 37 °C (Figure 2A). Crosslinking was indicated by increased smearing in the gel lanes, the disappearance of Ara h 1, which is found at about 64 kDa (Wichers and others 2004), the disappearance of other minor bands, and the accumulation of HMW protein polymers at the gel interface (Figure 2A, lanes 8 to 10). This pattern was not observed in control, nontreated, PF dispersions (lanes 2 to 5, Figure 2A). TGase activity was also measured in the presence of AP (1%) (Figure 2B); however, the degree of polymerization was reduced. Smearing as a result of TGase crosslinking was less prevalent in PF + AP dispersions after a 6-h treatment (Figure 2B, lanes 7 to 9 compared to lanes 2 to 4) although the formation of HMW polymers was still seen after a 24-h period (Figure 2B, lane10). Also, Ara h 1 was not visible after an extended treatment with TGase (Figure 2B, lane 10). Of particular interest was the observation that Ara h 2, with a relative molecular weight of about 20 kDa (Koppelman and others 2001), and a polypeptide of Ara h 3 with a relative molecular weight of

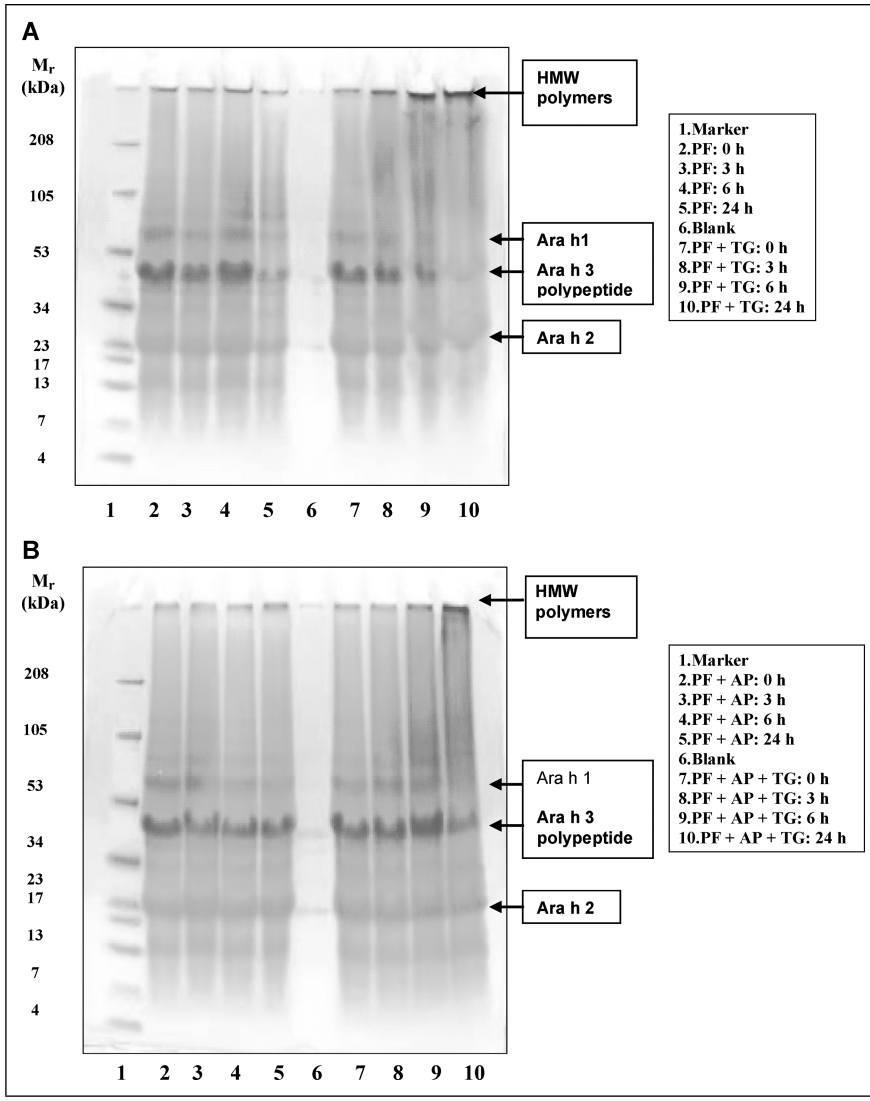

Figure 2—TGase polymerization of (A) 20% PF (w/w) and (B) 20% PF (w/w) + 1.0% AP (w/w), pH 8.0 at 0, 3, 6, and 24h at 37 °C. Approximately 30 μg of protein was loaded per lane.

about 45 kDa (Koppelman and others 2003), did not appear to be crosslinked to the same extent as Ara h 1 under these experimental conditions.

Quantitative measurements of protein crosslinking were determined using OPA assays, a testing method based on the loss of free amino groups. OPA data revealed approximately 40% crosslinking in TGase-treated PF dispersions that lacked AP (1%) compared to 20% for those containing the carbohydrate after a 6-h incubation period at 37 °C (Figure 3). Crosslinking percentages were determined based on the relative change in A_{340} nm in the presence and absence of TGase.

The decreased rate of TGase crosslinking in PF dispersions containing AP may be attributed to electrostatic interactions between PF protein constituents and AP, causing limited accessibility of noncovalently linked [PF protein-AP] conjugates to the enzyme's catalytic site. Indeed, polysaccharides have been shown to reduce *in vivo* and *in vitro* protein digestibility by various proteases, and numerous mechanisms have been put forth to explain this reduction in proteolytic activity, including direct protease–carbohydrate or carbohydrate–substrate interactions (Mouecoucou and others 2004). Protein-polysaccharide complexes can be formed as a result of electrostatic interactions and/or hydrogen bonding between the negatively charged carboxylic groups present in the polysaccharide and positive amino acid residues, such as lysine and arginine. In a separate report, pectin addition enhanced TGase crosslinking of soy proteins (Mariniello and others 2003), in which case electrostatic interactions between pectin and soy were reported to promote a preferred conformation of the soy protein substrate, resulting in increased TGase polymerization. The differing TGase response in these two systems might be attributed to the different types of pectins used in these experiments. In the current work, AP was added to PF dispersions while apple pectin USP was utilized in the work of Mariniello and others (2003). Also, the protein substrates were also obviously different, although soy proteins and peanut proteins share many similarities (Koppelman and others 2003).

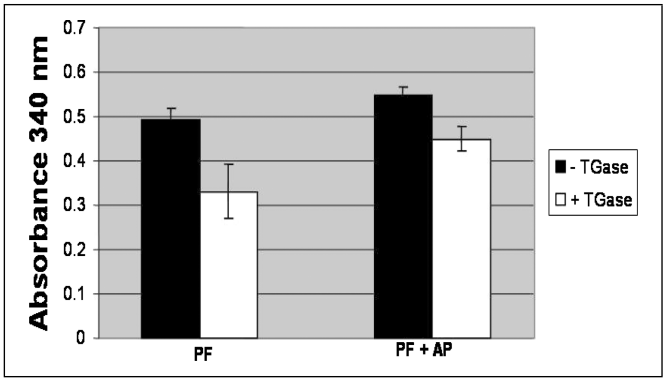

Figure 3 — OPA assay results for 20% PF dispersions (w/w) ± 1.0% AP (w/w) ± TGase, pH 8.0, after reaction at 37 °C for 6 h

Rheological testing was conducted at a concentration of 0.5% (w/w) AP, as the addition of 1% AP resulted in dispersions that were too viscous for rheological analyses. Small-strain data were obtained by continuously monitoring the viscoelastic changes that occurred under controlled heating conditions. Previously, gelation was observed for equivalent PF dispersions (no TGase and/or AP) under similar testing conditions, and the gel point was empirically defined as the point at which the storage modulus (G′) crossed over the loss modulus (G″) (Davis and others 2007). For the current experiments, gelation occurred at temperatures above 78 °C (Figure 4B) using samples treated with TGase, whereas control samples typically formed gels at about 68 °C (Figure 4A). Similar

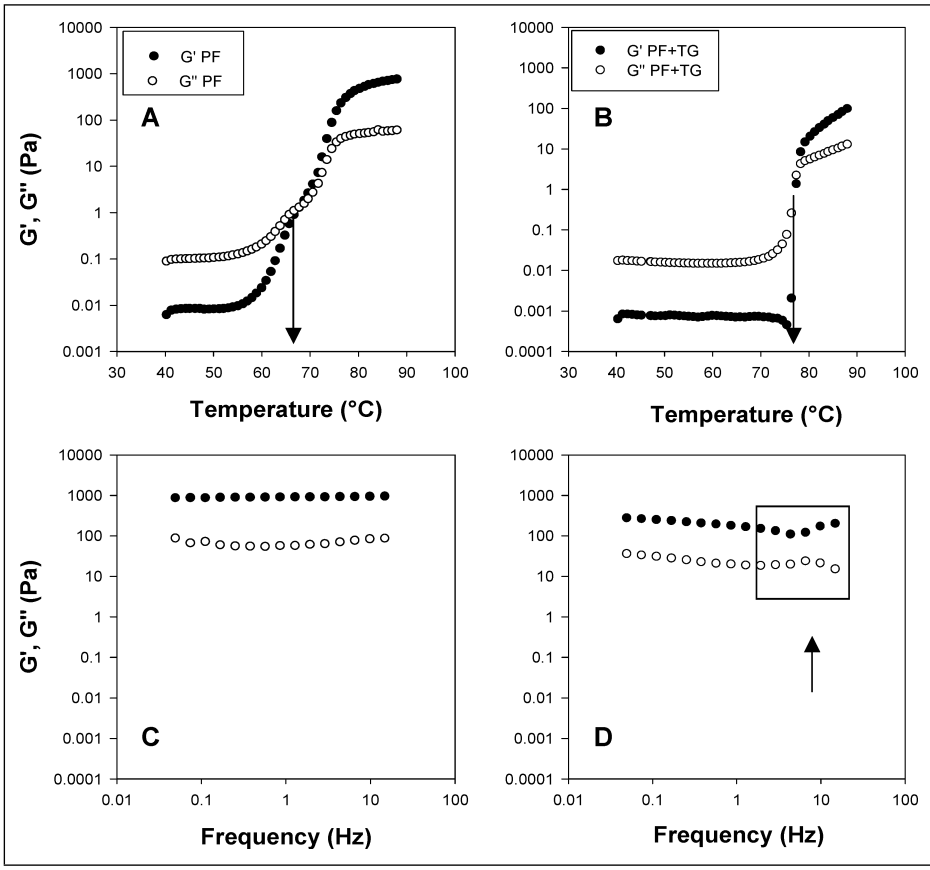

Figure 4 — Small-strain, oscillatory testing of PF ± TGase dispersions; stress = 1.5 Pa. PF dispersions were prepared as 20% total solids (w/w) and adjusted to pH 8.0. (A) and (B) represent heating ramps (1 °C/min) at a constant frequency of 0.1 Hz after a 7-h preincubation in the absence and presence of TGase at 37 °C, respectively. (C) and (D) depict frequency sweeps at a constant temperature of 90 °C after a similar incubation time. PF dispersions were prepared as 20% total solids (w/w) and adjusted to pH 8.0. Arrows were added to aid in data interpretation.

trends were noted in nontreated WPI solutions as compared to those crosslinked with TGase (Truong and others 2004). As previously discussed, these findings might be explained by the extensive crosslinking of the protein substrates such that adequate unfolding and network development of the PF matrix was compromised. Accordingly, other researchers have proposed that extensive crosslinking via TGase may limit exposure and hydrophobic interactions among whey proteins during heat denaturation (Eissa and Khan 2006; Eissa and others 2006). Ultimately, this process resulted in decreased gelation properties with respect to WPI and β-lactoglobulin as a result of these limited hydrophobic associations. Therefore, we hypothesize that a similar phenomenon may be occurring within these PF dispersions upon heating (Figure 4).

Dynamic oscillatory frequency sweeps at 90 °C were used to further characterize the small-strain rheological characteristics of PF ± TGase (Figure 4C and 4D). G′ was greater than G″ by roughly one order of magnitude across the frequency range tested in untreated PF dispersions (Figure 4C). These responses were essentially frequency independent, indicative of an established gel network (Steffe 1996). G′ and G″ were slightly higher in control PF dispersions compared to the enzyme-treated dispersions across this frequency range, indicative of a more rigid structure. PF dispersions treated with TGase displayed some frequency dependence especially at higher frequencies, also indicative of a weaker structure (Figure 4D).

Small-strain rheological data were collected over the heating range 40 to 90 °C for PF dispersions ±AP ± TGase as summarized in Figure 5. The addition of AP (Figure 5B) to the PF dispersion (Figure 5A) resulted in an increased viscosity that was most notable at temperatures below about 60 °C. These data are in agreement with those of previous researchers who reported that pectin, protein, and cellulose interactions affect rheological properties and that the addition of soluble polymeric molecules, such as pectin, increased solution viscosity (Brown and Stein 1977; Hoskins and others 1996). A more gradual transition from a viscous fluid to primarily an elastic gel was also observed in the PF + AP dispersion (Figure 5B) compared to the PF dispersion alone (Figure 5A). As discussed earlier, TGase treatment of PF dispersions shifted the gelling point to higher temperatures (Figure 5A and 5C). A similar increase in gel point was observed in PF + AP dispersions after TGase crosslinking (Figure 5B and 5D), although this shift was less obvious compared to enzyme-treated dispersions lacking AP (Figure 5A and 5C). This smaller shift in gelation temperature may be explained by the more limited degree of polymerization that occurred in PF dispersions prepared with AP. Regardless, the addition of TGase to either PF or PF + AP dispersions resulted in an increased gelation temperature.

Under large-strain deformations at a continuous shear rate of 50 per second, PF + AP dispersions were more viscous than PF dispersions, especially under low heating conditions (<70 °C; Figure 6). Samples treated with the enzyme for 6 h prior to rheological analysis were less viscous, both in the presence and the absence of AP (Figure 6B). This finding is in agreement with the recently published work by Clare and others (2007) and small-strain oscillatory analyses presented herein (Figure 4 and 5). At higher temperatures (about 77 to 90 °C), the apparent viscosity of dispersions without TGase was not stable at a shear rate of 50 per second. Perhaps, this instability might be due to the breakdown and reformation of the protein network in the presence of the shear field (Figure 6). Again, the decreased rate of TGase crosslinking observed in PF + AP dispersions (Figure 2B) as compared to PF dispersions (Figure 2A) may be attributed to the increased viscosity of the PF–AP dispersion, as

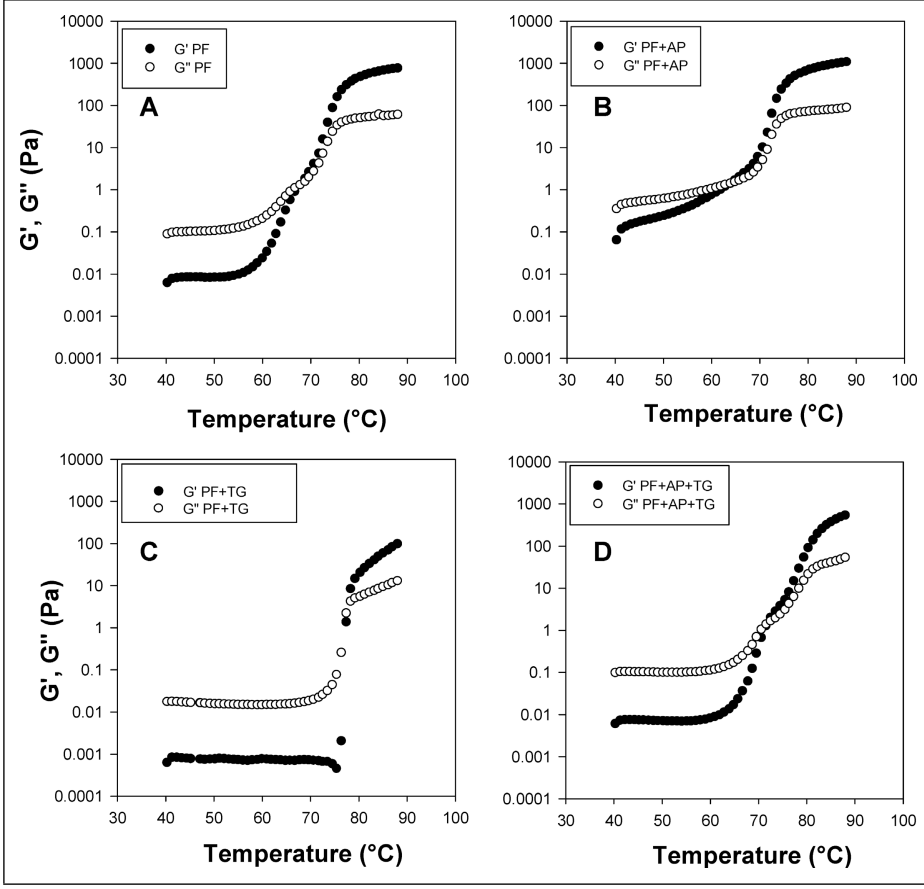

Figure 5—Small-strain, constant oscillatory testing of: A = PF, B = PF + AP, C = PF + TGase, and D = PF + AP + TGase; stress = 1.5 Pa, frequency = 0.1 Hz, heating ramps (1 °C/min). Rheological testing began immediately after the PF dispersions were treated with TGase for 7 h at 37 °C. Control, nonenzyme treated dispersions were also analyzed. PF dispersions were prepared as 20% total solids (w/w) ± 0.5% AP (w/w) and adjusted to pH 8.0.

thickened fluids slow the diffusion of both substrates and enzyme (Larsen and others 1994).

The WHC of PF dispersions ± AP ± TGase (24-h incubation) is summarized in Table 1. In other studies, a positive correlation was seen between the WHC of proteins and increased viscosity (Idris and others 2003; Khalid and others 2003; Ragab and others 2004). Accordingly, the increased viscosity that was observed in PF dispersions containing AP, both in the presence and the absence of TGase (Figure 6), correlated with an increased WHC of PF dispersions containing AP (Table 1). Previous researchers have also noted that the addition of TGase improved the WHC in a variety of prepared foods (Zhu and others 1995; Kuraishi and others 2001). Likewise, the addition of TGase to these PF dispersions, both in the presence and the absence of AP, significantly ($P < 0.001$) increased WHC after a 24-h incubation period (Table 1); however, no significant differences were noted among PF ± AP dispersions after crosslinking was accomplished for 6 h (data not shown). Furthermore, there was no correlation between WHC and viscosity upon the addition of TGase. Therefore, these approaches may be used in future applications to manipulate the WHC in various peanut-based food ingredients.

Table 1 — Water holding capacity of PF ± TGase ± AP dispersions after incubation for 24 h at 37 °C. PF dispersions were prepared as 20% (w/w) ± 0.5% AP (w/w) and adjusted to pH 8.0

Peanut flour sample	Water holding capacity[a] (g water held/g solid)
PF	5.64 ± 0.10[b]
PF + TGase	6.05 ± 0.05[c]
PF + AP	7.04 ± 0.05[d]
PF + AP + TGase	7.60 ± 0.03[e]

[a]Mean ± standard deviation. Means in a column followed by a different letter are significantly different ($P < 0.001$).

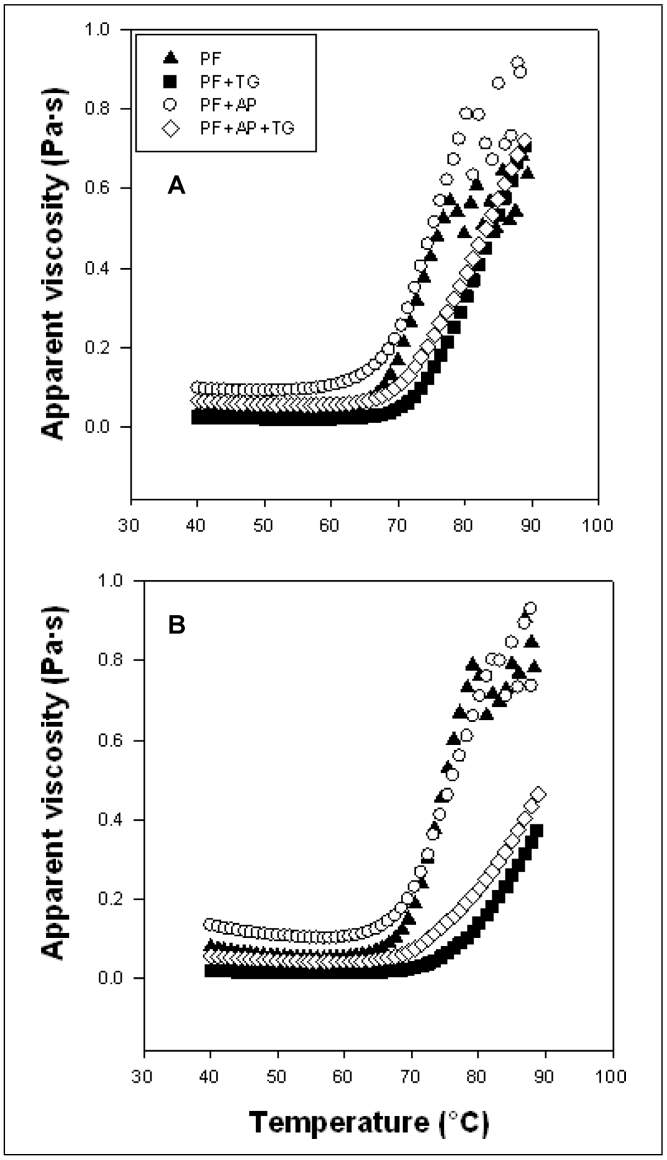

Figure 6 — Rotational viscosity testing of PF, PF + TG, PF + AP, and PF + AP + TG. The shear rate was 50 per second. PF dispersions were prepared as 20% total solids (w/w) ± 0.5% AP (w/w) and adjusted to pH 8.0. The heating rate was 1 °C/min. A = dispersions at 0 h. B = dispersions after a 6-h preincubation at 37 °C in the presence and absence of TGase.

Conclusions

Enzymatic polymerization of PF dispersions was achieved in the presence and absence of AP; however, polymer formation was more rapid in PF dispersions lacking AP. OPA assays revealed approximately 40% protein coupling of PF dispersions + TGase as compared to approximately 20% protein coupling of PF + TGase + AP. PF + AP + TGase supernatant fractions showed higher protein content than PF + TGase protein dispersions. TGase-treated PF dispersions formed gels at higher temperatures compared to control dispersions. The apparent viscosity of PF + TGase and PF + AP + TGase dispersions was decreased, whereas the WHC was increased after treatment with TGase after 24 h at 37 °C. The additive effects of TGase polymerization of PF ± AP dispersions resulted in the modification of functional characteristics such as gelation temperature, apparent viscosity, and WHC. These data suggest potential applications of polymerized PF dispersions in peanut-based food products, including protein bars, shakes, and value-added baked goods.

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